Effects of Sample Holding Time on Concentrations of Microorganisms in Water Samples

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ABSTRACT: This research investigated the effects of extending the holding time of samples for microbial analysis beyond the standard of 24 hours for purposes such as watershed characterization. Experiments were conducted with both sanitary wastewater and stormwater samples. The refrigerated samples (4 °C) were held for up to 9 days before being analyzed for two pathogens (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) and five indicator organisms (total coliform, fecal coliform, fecal streptococcus, enterococcus, and *Escherichia coli*) by membrane filtration. The concentrations (as colony-forming units per 100 mL) were normalized by $\log_{10}$ transformation and used in subsequent statistical analysis testing for significant differences. The results suggested that the concentrations of microorganisms in water samples analyzed on days 1 and 2 did not vary significantly in 8 of 13 analyses. The results of a field study concluded that the concentration of fecal coliform did not change significantly between 7 hours holding time and greater than 24 hours holding time for fecal coliform. *Water Environ. Res.,* **76,** 67 (2004).

KEYWORDS: holding time, stormwater, sanitary wastewater, indicator organisms, pathogens, watershed.

Introduction

The U.S. Environmental Protection Agency's (U.S. EPA's) 1998 National Water Quality Inventory Report to Congress showed that approximately 40% of state-assessed U.S. streams, lakes, and estuaries are failing to support the criteria for locally designated uses such as fishing and swimming (U.S. EPA, 2000). High bacteria concentrations in stormwater runoff from agricultural and urban areas are a leading cause of the failure to meet designated-use criteria (U.S. EPA, 2000). Stormwater discharges occur from as many as 1.2 million municipal, industrial, commercial, and retail sources in the United States. Pollutant loads from these nonpoint sources are not commonly evaluated through direct sampling efforts, but are estimated using receiving water samples as a surrogate. Using receiving water surrogate samples confounds the data analysis and introduces uncertainty. A key difficulty with direct sampling is the short sample holding times associated with microbiological analyses.

Depending on the end use of the sample analysis, the allowable sample holding time varies. Some samples, such as nonpotable water for compliance purposes, must be analyzed within a few hours (6 hours) for the results to maintain legal integrity, while other samples intended for applications with fewer stringent demands may be analyzed after a longer holding time (24 hours) (APHA et al., 1998). Current regulations allow drinking water samples to be analyzed within 30 hours. McDaniels et al. (1985) studied the effects of holding time on enumeration in drinking water samples and found that coliform in samples held for 24 and 30 hours at 5 °C showed losses as great as 23 and 33%, respectively. Lonsane et al. (1967) observed that the coliform density decreased with the storage time at both room and refrigerator temperatures. They also found that with marginally polluted waters, the differences were not significant between the original counts and the counts obtained after storing them for 24, 48, and 72 hours at both room and refrigerator temperatures. Standridge and Lesar (1977) examined 28 samples of heavily polluted water with initial coliform counts between 10^2/mL and 10^6/mL and found little change after storage at 2 to 4 °C for 24 hours. Experiments conducted by Robertson and Gjerde (2000) showed that extending holding time fivefold had no effect on analytical results for *Cryptosporidium* and *Giardia* from raw water, although U.S. EPA's method 1623 stipulates that the filtration, elution, and concentration must be completed within 72 hours of sample collection (U.S. EPA, 1999).

Despite this supporting evidence, the allowable holding time is short even for nonregulatory samples other than regulatory-based sampling efforts. Conceptually, the holding time can be extended indefinitely if analytical results do not vary beyond generally accepted confidence levels and data quality meets intended purposes. The short holding time for microorganism analysis can eliminate most advantages of using automated sampling techniques across several sites, significantly increasing sampling costs as dedicated sampling personnel, who are restricted to the accuracy of the weather forecasts, wait for the opportunity to sample a rain event. Similarly, laboratory throughput capacity limits the sampling intensity to the number of analyses that can be completed within the designated holding times.

Extending sample holding times could possibly reduce the cost associated with sample collection or increase the breadth of the study by decreasing overtime operations or increasing the number of samples analyzed. However, sample holding and handling techniques must have some upper time limit to ensure the analysis is complete before the natural biological processes alter the result sufficiently to change the conclusions. Watershed characterization studies, research projects, preliminary screening efforts, and other specific applications can accept higher uncertainty levels than specific enforcement actions tied to established regulatory guidelines. Therefore, it should be possible to extend the holding time based on statistical confidence levels.

Objectives

This study investigated whether lengthening the holding time of samples significantly affects the measured concentrations of microorganisms. This effort tested the hypothesis that the analytical result of a sample analyzed for total coliform, fecal coliform, fecal streptococcus, enterococcus, *Escherichia coli*, *Pseudomonas aeru-*

ginosa, and *Streptococcus aureus* after an extended holding time is statistically equivalent to a sample analyzed on the first day at the 95% confidence level. The first data evaluation specifically evaluated the ability to add 1 day to the holding time. If the 1-day extension was feasible, the ability to further extend was evaluated. The subsequent evaluations, as secondary evaluations, are targeted to have a lower statistical power and include fewer replicate analyses.

Methods

The U.S. EPA's Urban Watershed Management branch completed a laboratory study in two phases. The first effort used a sanitary wastewater sample as the source of the target organisms. The high concentrations in sanitary wastewater would make it easier to detect changes in concentration, assuming the organisms follow a pseudo-first-order decay process. The first-order decay of microorganisms can be written as

$$N_t = N_0 \exp(-kt) \quad (1)$$

Where

N_t = concentration of an organism at time t (colony-forming units [CFUs]/100 mL),
N_0 = concentration of an organism at day 1 (CFU/100 mL),
k = decay constant under the ambient conditions (1/d), and
t = time (d).

Equation 1 can be rewritten in the following form:

$$\log N_t = \log N_0 - kt \quad (2)$$

During the second phase, a stormwater sample was analyzed to reveal matrix effects that could give differing results for stormwater.

Additionally, a watershed assessment study sponsored by U.S. EPA and peer reviewed by the Water Environment Research Foundation was conducted on the Middle Chattahoochee River in Columbus, Georgia. During the study, a nonconventional sample holding time of up to 24 hours (versus the 6-hour standard) was used for fecal coliform analysis. Extended holding times were necessary because of the difficulties associated with wet-weather sampling, laboratory availability, and the number of samples generated during each event. The project peer-review committee raised concerns related to the extended holding time. A field study was subsequently conducted to assess the effects of a 24-hour holding time on fecal coliform concentrations under several conditions.

Sample Collection. *Sanitary Wastewater.* A 1-L (0.26-gal) sanitary wastewater grab sample was collected from the influent at a wastewater treatment facility in Ringwood, New Jersey, using a precleaned (method 9040, APHA et al., 1998) high-density polyethylene (HDPE) container on April 17, 2000. The sample was placed in a cooler with ice and driven to the laboratory.

Stormwater. An automatic sampler (model 900 max, American Sigma, Loveland, Colorado) collected a flow-weighted stormwater sample from a 0.38-m (15-in.) diameter concrete storm sewer outfall. The storm sewer drains a small, slightly sloping, high-density residential area in Monmouth County, New Jersey. Earlier evaluations following the procedures developed by Pitt et al. (1993) showed the storm sewer was unlikely to have sanitary cross connections. The automatic sampling began when the flowing water depth in the storm sewer reached 3 cm (1 in.). The sampler added one 1-L sample to the container after each 1350 L (357 gal) of stormwater flow was measured by the attached flow meter (model 960, American Sigma). A calibrated peristaltic pump transferred the samples to a precleaned 19-L (5-gal) HDPE container. The sample was collected during a rain event on July 10, 2000, that produced 1.8 mm (0.07 in.) of total rainfall over 74 minutes. Rainfall was recorded using a tipping bucket rain gauge (model RGD-04, Environmental Sensors, Inc., Escondido, California) positioned near the sampler within the drainage area. The runoff was slightly acidic (pH = 6.03 to 6.86), with a conductivity of 0.1 to 0.2 mS and a temperature of 20.5 to 23.8 °C. The gauge recorded no rain at the site during the preceding 140 hours. The nearly 6-day dry period was believed to be sufficient for normal pollutant buildup processes to reach equilibrium, assuming an exponential buildup as proposed by Sartor and Boyd (1972). The sample was recovered, placed in a cooler with ice, and transported to the laboratory for processing.

Because of nonquantitative data linked to inappropriate sample dilutions (below the detection limit or too numerous to count) for several organisms, a second stormwater sample was collected during a rain event on April 11, 2001. The event produced 9.4 mm (0.37 in.) of total rainfall over 10.5 hours. The rain gauge recorded no measurable rainfall during the previous 46 hours. Water quality parameters such as pH, conductivity, and temperature were not measured during this sampling event.

Sample Analysis. The incoming sample was stirred well and divided into subsamples for analysis on designated days. The first subsample was analyzed as soon as possible on the day of collection (day 1) (i.e., within 24 hours of collection). The remaining subsamples were held in refrigerated (4 °C) storage until the designated day for analysis. The samples were analyzed for two pathogens (*P. aeruginosa* and *S. aureus*) and five indicator organisms (total coliform, fecal coliform, fecal streptococcus, enterococcus, and *E. coli*). Sample analysis followed membrane filtration methods (methods 9222B, D, and G; method 9230C; and method 9213B and E) outlined in *Standard Methods* (APHA et al., 1998). Each sample was sequentially diluted using three dilution factors based on previous analyses of samples from the same source. Dilutions used at least 10 mL in each sequential dilution step. The dilution factors were selected to obtain the desired colony count on each incubated plate. Two to five diluted subsamples were analyzed. For sanitary wastewater, four diluted subsamples were analyzed on days 1 and 2 and two were analyzed on subsequent days. For stormwater, five diluted subsamples were analyzed on day 1, four on day 2, and two on subsequent days. All results were normalized to give concentrations in colony-forming units per 100 mL. Blanks were run before and after each analytical set. Verification was performed on 10 colonies for each organism according to *Standard Methods.* Relative standard deviations were set at no more than 70%. The experiment was repeated on a sample collected on April 11, 2001, for enterococcus, *E. coli*, *P. aeruginosa*, and *S. aureus*. The study was conducted over 3 days.

Data Analysis. The measured concentration from the analyses yielding plate counts in the desired range was used. If no dilution provided plates with the desired colony counts or if more than one dilution provided counts in the desired range, then the dilution set(s) most closely meeting the requirements were used. Nonquantitative data were excluded in the data analysis. Concentrations were transformed to common logarithm ($\log_{10}$) to convert the data to a normal distribution for analysis. A standard analysis of variance (ANOVA) on the log-transformed concentration was used to detect significant differences in the original concentration as well as the concentration on subsequent days. The difference between concen-

Table 1—Geometric mean microorganism concentrations determined in sanitary wastewater.[a,b]

Analysis	Analysis day (Elapsed time in hours from sample collection)						Significance (ANOVA of days 1–8)
Total coliform	1 (3.5) 6.3×10^7	2 (27) 5.3×10^7 ($p = 0.21$)	3 (51) 2.9×10^7	4 (74) 3.0×10^7	6 (121.5) 4.4×10^7	NA	Significant ($p = 0.025$)
Fecal coliform	1 (4) 1.4×10^6	2 (27.3) 1.0×10^6 ($p = 0.49$)	3 LE	4 (75.8) 0.5×10^6	6 (124) 0.4×10^6	7 (147.5) 0.6×10^6	Significant ($p = 0.001$)
Fecal streptococcus	1 (7.5) 1.8×10^5	2 (30.5) 0.9×10^5 ($p = 0.13$)	3 (54) 1.6×10^5	4 (77) 3.0×10^5	6 (125.5) 1.5×10^5	8 (173.5) 0.7×10^5	NS ($p = 0.054$)
Escherichia coli	1 (8) 1.5×10^5	2 (31) 3.2×10^5 ($p = 0.01$)	3 (54.5) 1.3×10^5	4 (77.5) 1.6×10^5	6 (126) 0.7×10^5	7 (148.5) 2.3×10^5	Significant ($p < 0.001$)
Pseudomonas aeruginosa	1 (8.5) 5.3×10^4	2 (31.5) 5.5×10^4 ($p = 0.65$)	3 (54.5) 5.0×10^4	4 (78) 5.5×10^4	6 (126.5) 5.9×10^4	8 (172.5) 3.7×10^4	NS ($p = 0.122$)
Staphylococcus aureus	1 (7) 3.6×10^5	2 (30) 3.8×10^5 ($p = 0.75$)	3 (54) 3.4×10^5	4 (76.5) 4.7×10^5	6 (125) 5.1×10^5	8 (172) 3.5×10^5	NS ($p = 0.082$)

[a] All concentrations are in colony forming units per 100 mL.
[b] LE = laboratory error; NA = not analyzed; NS = not significant (p is set at ≤ 0.05).

trations was regarded as statistically significant if the calculated probability value, *p,* was less than 0.05. The statistical analysis was completed using Statistica '98 (StatSoft, Inc., Tulsa, Oklahoma). Linear regression analyses were done using SigmaStat software (version 2.0, SPSS, Inc., Chicago, Illinois) on the log-transformed daily means calculated for the replicate analysis of subsamples. Linear regression analysis was performed to determine whether the change in microorganism concentrations follows the first-order

Table 2—Geometric mean microorganism concentrations determined in stormwater.[a,b]

Analysis	Analysis day (Elapsed time in hours from sample collection)						Significance (ANOVA of days 1–9)
First sampling event							
Total coliform	1 (7.5) 0.8×10^5	2 (34.5) 0.5×10^5 ($p = 0.53$)	3 (52) 5.7×10^5	7 (144.3) 7.8×10^5	8 (167.8) 1.3×10^5	9 (191.8) 1.1×10^5	Significant ($p = 0.046$)
Fecal coliform	1 (3.8) 1.3×10^4	2 (34.3) 1.3×10^4 ($p = 0.86$)	3 (51.3) 3.9×10^4	6 (144.5) 1.5×10^4	7 (167.8) 1.1×10^4	8 (191.8) 0.3×10^4	Significant ($p < 0.001$)
Fecal streptococcus	1 (9.3) 1.0×10^3	2 (34.5) 8.1×10^3 ($p < 0.001$)	3 (50.3) 2.0×10^3	6 (146.8) 0.6×10^3	7 (167.8) 0.5×10^3	8 (191.8) 0.2×10^3	Significant ($p = 0.008$)
Second sampling event							
Enterococcus	1 (2) 2.7×10^2	2 (26) 4.0×10^2 ($p = 0.04$)	3 (50) 2.5×10^2	NA	NA	NA	Significant ($p = 0.005$)
Escherichia coli	1 (1.5) 3.9×10^3	2 (25.5) 5.5×10^2 ($p < 0.001$)	3 (49.5) 2.8×10^2	NA	NA	NA	Significant ($p < 0.001$)
Pseudomonas aeruginosa	1 (2) 6.1×10^2	2 (26) 6.8×10^2 ($p = 0.16$)	3 (50) 8.1×10^2	NA	NA	NA	Significant ($p = 0.022$)
Staphylococcus aureus	1 (1.5) 2.6×10^4	2 (25.5) 7.4×10^4 ($p < 0.001$)	3 (49.5) 9.7×10^4	NA	NA	NA	Significant ($p < 0.001$)

[a] All concentrations are colony-forming units per 100 mL.
[b] NA = not analyzed; NS = not significant (p is set at ≤ 0.05).

Table 3—Field study holding time analytical results.

Sample analysis		Fecal coliform concentrations, *C* (CFU/100 mL)					
		Refrigerated container		Iced container		Ambient container	
Date	Time	*C*	Log *C*	*C*	Log *C*	*C*	Log *C*
11/22/99	9:00 AM	435	2.638	435	2.638	435	2.638
11/22/99	10:00 AM	405	2.607	320	2.505	450	2.653
11/22/99	11:00 AM	425	2.628	530	2.724	400	2.602
11/22/99	12:00 Noon	285	2.455	290	2.462	480	2.681
11/22/99	1:00 PM	295	2.470	320	2.505	330	2.519
11/22/99	2:00 PM	355	2.550	280	2.447	240	2.380
11/22/99	3:00 PM	365	2.562	310	2.491	260	2.415
11/22/99	4:00 PM	330	2.519	380	2.580	320	2.505
Mean (0–7 h)		*358*	*2.554*	*350*	*2.544*	*354*	*2.549*
11/23/99	8:30 AM	335	2.525	420	2.623	380	2.580
11/23/99	9:43 AM	260	2.415	330	2.519	270	2.431
11/23/99	10:32 AM	305	2.484	290	2.462	340	2.531
11/23/99	11:32 AM	320	2.505	360	2.556	300	2.477
11/23/99	12:50 AM	315	2.498	340	2.531	220	2.342
Mean (24–28 h)		*306*	*2.486*	*345*	*2.538*	*297*	*2.472*

decay model at 4 °C and whether the kinetic constant differs significantly from zero.

Results

Sanitary Wastewater. Table 1 lists the geometric mean concentrations measured on each day with the elapsed time since the sample collection. The initial concentrations are within the range expected from the literature (Metcalf & Eddy, 1991). Concentrations of total coliform, fecal coliform, and fecal streptococcus were lower on day 2 than on day 1. Measured concentrations of *E. coli*, *P. aeruginosa*, and *S. aureus* were higher on day 2 than on day 1. Other than *E. coli*, the differences are not significant. Although only one data value is available for fecal coliform on the second day, it cannot be excluded from the statistical population measured on the first day.

The measured concentrations did not vary significantly during the 8-day evaluation period for fecal streptococcus, *P. aeruginosa*, or *S. aureus*. The variation in the *E. coli*, total coliform, and fecal coliform concentrations is significant.

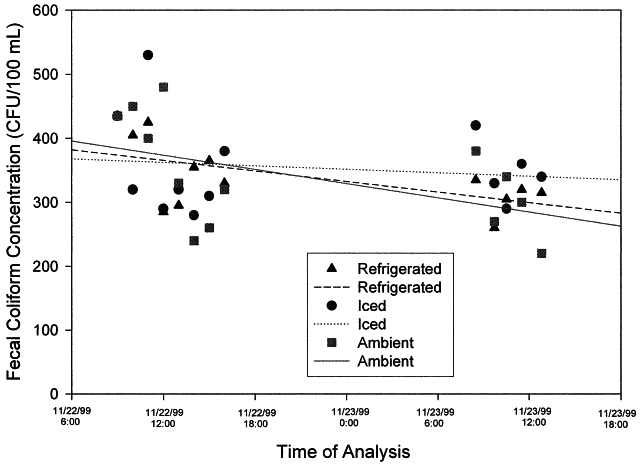

Figure 1—Field study results.

Linear regression analysis was performed on the log-transformed daily means. The *p*-values show no relationship between density and time over this range under these conditions. Computed *p*-values varied from 0.057 for fecal coliform to 0.595 for *E. coli*.

Stormwater. Table 2 lists the geometric mean concentrations measured on each day. Enterococcus was added for the stormwater evaluation. The initial concentrations are within the range reported in the literature (Thomann and Mueller, 1987). The concentrations of all organisms except for total coliform, fecal coliform, and *Escherichia coli* are higher on day 2 than on day 1. Concentrations of fecal coliform were almost the same on both days. *E. coli* and fecal streptococcus changed nearly one order of magnitude; both of these seem anomalous. The concentrations of fecal streptococcus, enterococcus, *E. coli*, and *S. aureus* changed significantly between days 1 and 2. The total coliform, fecal coliform, and *P. aeruginosa* analysis are not statistically different on the first and second day.

The variation in the concentration of all of the microorganisms was statistically significantly over the duration of testing. Concentrations of total coliform, *P. aeruginosa*, and *S. aureus* increased on subsequent days. Concentrations of other organisms decreased on subsequent days. The *E. coli* concentration decreased by 14-fold on day 3.

Linear regression analysis was performed on the log-transformed daily means and the *p*-values indicate that there is no significant relationship between density and time. The *p*-values ranged from 0.063 for fecal streptococcus to 0.894 for enterococcus.

No consistent patterns or changes were evident between organisms or matrices. For sanitary wastewater, concentrations of total coliform, fecal coliform, and fecal streptococcus decreased between days 1 and 2, and concentrations of *E. coli*, *P. aeruginosa*, and *S. aureus* increased. For stormwater, concentrations of all of the organisms increased except for total coliform, fecal coliform, and *E. coli* between days 1 and 2. On subsequent days, concentrations of organisms in sanitary wastewater decreased except for *E. coli*.

Concentrations of fecal coliform, fecal streptococcus, enterococcus, and *E. coli* in stormwater decreased, while concentrations of total coliform, *P. aeruginosa*, and *S. aureus* increased.

Field Study. Approximately 27 L (7 gal) of water was collected from a marina at Lake Oliver in Georgia in the slough of a suburban feeder creek on the morning of November 22, 1999. The sample was split into three containers of approximately equal volume. The sample was immediately tested for fecal coliform. Container 1 was placed in a laboratory refrigerator and maintained at a temperature of 4 °C. Containers 2 and 3 were each set inside the sample carousel of an automatic sampler. Both samplers (and sample containers) were set outside. Container 2 was iced; container 3 was not iced and was kept under ambient conditions. Each container was sampled for fecal coliform and temperature every hour (during normal business hours) for the next 28 hours. The results are summarized in Table 3 and Figure 1.

The log-transformed concentrations were tested using standard ANOVA techniques. The mean concentration of the first 7 hours of holding time was compared to the mean concentration of the greater-than-24-hour holding time for all three conditions. The results show that there is not a statistically significant difference in measured concentrations attributable to holding time under tested conditions. The results also show that there is not a statistically significant difference among the three containers. The fecal coliform concentration measured during the first 7 hours of holding time was slightly greater than concentrations measured beyond the 24-hour holding time.

Discussion

This effort tested the hypothesis that the analytical result of a sample analyzed for microorganisms in water samples after an extended holding time is statistically equivalent to a sample analyzed within the regulatory holding time of 24 hours at the 95% confidence level. For sanitary wastewater, except when analyzing for *E. coli*, the sample holding time can be extended beyond 24 hours without affecting the data quality. For stormwater, the sample holding time can be extended beyond 24 hours for total coliform, fecal coliform, and *P. aeruginosa*. As such, application of the hypothesis depends on both the microorganism and matrix of the sample. It further suggests that when looking at multiple organisms in a sample, a priority can be established to reduce potential negative effects of the time extension. While analysis within the holding time has clear advantages and should be used when possible, some targeted extensions are possible when supported by sample-specific evaluations.

Other investigators have studied the effect of holding time only with coliforms. Lonsane et al. (1967) observed that the concentration decreased with storage time and the differences were not significant for marginally polluted waters. Standridge and Lesar (1977) examined 28 samples of heavily polluted water with initial coliform counts between 10^2/mL and 10^6/mL and found little change after storage at 2 to 4 °C for 24 hours. These results for total and fecal coliform in both sanitary wastewater and stormwater agree with those of other investigators.

Conclusions

In comparing one sanitary sewage sample and two stormwater samples, concentrations of all microorganisms were found to be one to two orders of magnitude higher in sanitary sewage than stormwater. The difference in results between the sanitary wastewater and stormwater suggests that the sample type influences the rate of concentration change. Linear regression analysis performed on the log-transformed daily means suggests that the change of organism concentration with time does not follow the first-order decay model at 4 °C or, alternately, the kinetic constant did not differ significantly from zero under these conditions. It also suggests that the temperature of 4 °C selected for this study is efficient at preserving the samples. These results also suggest that, when necessary, the holding time can be extended beyond 24 hours for organisms such as total coliform, fecal coliform, and *P. aeruginosa* without affecting data quality. The results of the field study further supported the conclusion that the sample holding time can be extended beyond 24 hours for fecal coliform.

The samples should be analyzed within the standard holding time; however, if conditions prevent all samples from meeting the holding time requirements, these results show how to select the order of analysis for multiple organisms. In this example, fecal streptococcus, enterococcus, *E. coli*, and *S. aureus* should be analyzed before the other organisms.

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